Physical Characterisation of the Rhamnogalacturonan and Homogalacturonan Fractions of Sugar Beet (*Beta vulgaris*) Pectin

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Abstract

Acid-extracted sugar beet *(Beta vulgaris)* pectin was subjected to enzymatic hydrolysis using fungal pectin methyl esterase (f-PME) and two endopolygalacturonanases (PGs I and II). From the hydrolysate, the RG-I fraction was separated and purified by chromatographic techniques. This RG-I fraction was shown to be of high weight average molar mass (188000 g/mol), but low intrinsic viscosity (36 ml/g), which is consistent with a random coil conformation (L_p = 1.4 nm).

The HG fraction was prepared by mild acid hydrolysis of acid extracted pectin. The HG fraction was found to have a relatively low weight average molar mass (20000 g/mol), but a rather high intrinsic viscosity (77 ml/g), which is consistent with the HG fraction being rigid in solution (L_p = 9.8 nm).

Lower molar mass pectins are richer in HG regions and pectins of higher molar mass are richer in RG-I regions. We conclude that the degradation of the HG region has an important impact on intrinsic viscosity, but less on molar mass and the inverse is true for the degradation of RG-I region. This has important consequences in terms of the functionality of sugar beet pectin molecules.

Key words:

Sugar beet *(Beta vulgaris)* pectin; endopolygalacturonase (endo-PG); rhamnogalacturonan (RG-I) region; acid hydrolysis; homogalacturonan (HG) region.

1. Introduction

Pectins are a complex family of heteropolysaccharides that constitute a large proportion of the primary cell walls of dicotyledons and play important roles in growth, development and senescence (Ridley, O'Neil, & Mohnen, 2001; Willats, McCartney, Mackie, & Knox, 2001). Pectic polysaccharides are made of several structural elements the important of which are the homogalacturonan (HG) and type I rhamnogalacturonan (RG-I) regions often described in simplified terms as the "smooth" and "hairy" regions respectively. The HG region is composed of (1→4) linked α-D-Gal*p*A residues that can be partially methylated at *C*-6 (Pilnik & Voragen, 1970) and possibly partially acetyl-esterified at *O*-2 and/or *O*-3 (Rombouts, & Thibault, 1986). The degree of methylation (DM) and the degree of acetylation (DAc) are defined as the number of moles of methanol or acetic acid per 100 moles of GalA. The degree of methylation in native pectins is generally in the order of DM ≈ 70-80; whereas degree of acetylation is generally much lower *e.g.* DAc ≈ 35 for sugar beet pectins (Rombouts, & Thibault, 1986). The RG-I region consists of disaccharide repeating unit [→4)-α-D-Gal*p*A-(1→2)-α-L-Rha*p*-(1→]$_n$ with a variety of side chains consisting of L-arbinosyl and D-galactosyl residues (Voragen, Pilnik, Thibault, Axelos, & Renard, 1995). It has been reported that GalA residues in the RG-I region are partially acetylated (Ishii, 1997; Perrone, Hewage, Thomson, Bailey, Sadler, & Fry, 2002) but not methylated (Komalavilas, & Mort, 1989; Perrone, *et. al.*, 2002). In the case of sugar beet pectin the neutral side chain sugars are substituted with ferulic acid (Fry, 1982; Rombouts, & Thibault, 1986) and there is evidence indicating that pectin chains can be dimerised via diferulic bridges (Levigne, Ralet, Quéméner, & Thibault, 2004a; Levigne, Ralet, Quéméner, Pollet, Lapierre, & Thibault, 2004b; Ralet, Cabrera, Bonnin, Quéméner, Hellin, & Thibault, 2005a). There are a number of different ways in which ferulic acid can dimerise the most common being: 5-5'; 8-O-4'; 8-5' cyclic and 8-5' non-cyclic dimers (Micard, Grabber, Ralph, Renard, & Thibault, 1997).

In order to characterise the physical and chemical properties of the type I rhamnogalacturonan region it is necessary to degrade the homogalacturonan region. This can be achieved in a controlled manner using polygalacturonanses (PGs). PGs catalyse the hydrolytic cleavage of the O-glycosyl bond of α-D-(1→4) polygalacturonate. This degradation proceeds in either a random (*endo*-PG, E.C. 3.2.1.15) or a terminal (*exo*-PG, E.C. 3.2.1.67) fashion. A large number of

fungal PGs have been purified and characterised and although they appear to have similar amino acid compositions (Mohamed, Christensen, & Mikkelsen, 2003) they have different chemical and physical properties. *Aspergillus niger* has been shown to posses at least seven *endo*-PGs (Benen, van Alebeek, Voragen, & Visser, 2003). Both methyl and acetyl groups have been shown to affect the mode of action of *endo*-PGs (Renard, & Jarvis, 1999a,b; Mohamed, *et. al*., 2003).

In the present study, two different *endo*-PGs were used in conjunction with a fungal pectin methyl esterase (f-PME) to generate the RG-I region of sugar beet pectin. The enzyme hydrolysate was fractionated by anion exchange and size exclusion chromatography and the physical and chemical properties of the resultant RG-1 fragment was characterised.

The homogalacturonan (HG) region was generated by mild acid hydrolysis of sugar beet pectin after saponification with sodium hydroxide at 4 °C in order to prevent β-elimination. The physical characteristics of the HG region were measured without any further purification.

2. Experimental

2.1. Materials

2.1.1. Enzymes

Recombinant fungal pectin methyl esterase from *A. aculeatus* (f-PME, E.C. 3.1.1.11, UniProt Q12535) and endopolygalacturonase I and II from *A. niger* (PG I and PG II, E.C. 3.2.1.15, UniProt P26213 and P26214) were provided by Novozymes (Bagsvaerd, Denmark). The purification of PGI and PGII are described in detail in Ralet, *et al.* (2008a) and Bonnin, *et al*. (2002a), respectively.

2.2. Methods

2.2.1. Acid extraction of sugar beet pulp (Pectin A)

The alcohol insoluble residue (AIR) (5 g) from sugar beet pulp was obtained as described in Levigne, Ralet, & Thibault (2002) in brief sugar beet roots were immersed in 3 litres of boiling ethanol (96%) and the slurry was filtered through G3 sintered glass and the insoluble material was repeatedly suspended in 70 % ethanol until the filtrate gave a negative reaction to the

phenol-sulphuric acid assay (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). The resultant AIR was then dispersed in 150 ml HCl at pH 1.0 and heated for 30 mins at 75 °C with agitation. The residue was separated through G3 sintered glass. The supernatant was adjusted to pH 4.5 with 2 M NaOH, concentrated under vacuum at 40 °C, extensively dialysed against distilled water and freeze-dried at condenser temperature of -55 °C and a pressure of 4 millibars (400 Pa) for 48 hours. The resultant pectin (yield 280 mg/g) will be referred to as pectin A. The RG-I and HG fractions of pectin A will prepared by enzymatic and acid hydrolysis, respectively and characterised in terms of molar mass, intrinsic viscosity and confirmation after purification where appropriate (see **Figure 1**).

2.2.2. Mild acid hydrolysis of pectin A

Pectin A (50 mg) was dissolved in distilled water at approximately 10 mg/ml and the pH was increased to pH 12 by the addition of 0.2 M NaOH. The mixture was incubated for 24 hours at 4 °C, after which the pH was lowered to pH 1 on addition of 2.5 M HCl. The resultant mixture was heated at 80 °C for 72 hours, during which a precipitate was formed. This precipitate has previously been shown to be the homogalacturonan region (Thibault, Renard, Axelos, Roger, & Crépeau, 1993). The precipitate (AHCl72) was separated by centrifugation and resolubilised by neutralisation with sodium hydroxide (1 M) prior to analysis by HPSEC.

2.2.3. Enzymatic hydrolysis of pectin A

Sugar beet pectin A (100 mg) was dissolved in 0.05 M sodium succinate buffer pH 4.5 at a concentration of 7.5 mg/ml overnight at 30 ºC with gentle agitation. The pectin solution was mixed with an enzymatic solution (in 0.05 M succinate buffer pH 4.5) containing f-PME, PG I and PG II. Enzymes were present 25.7, 2.1 and 2.1 nkat/ml respectively in the final reaction mixture. The reaction mixture containing 4.3 mg pectin/ ml was incubated at 30 °C for 24 hours.

2.2.4. Analytical

Galacturonic acid and neutral sugar (expressed arbitrarily as arabinose) contents were determined in triplicate by the automated m-hydroxbiphenyl (Thibault, 1979) and orcinol methods (Tollier, & Robin, 1979), respectively, the latter being corrected for interfering galacturonic acid.

Individual neutral sugars were obtained by hydrolysis with 2 M trifluoroacetic acid at 121 °C for 2 hours and converted to their corresponding alditol acetates (Blakeney, Harris, Henry, & Stone, 1983). These alditol acetates were subsequently analysed by gas chromatography using myo-inositol (0.5 mg) as an internal standard on a DB-225 fused-silica capillary (30m x 0.32 mm i.d.) column (J&W Scientific, Courtaboeuf, France) mounted in a DI 200 chromatograph (Delsi Nermag Instruments, Argenteuil, France) with hydrogen as the carrier gas at a constant temperature of 220 ºC.

Phenolic acids were determined by HPLC after saponification and extraction. Pectin A (8.1 mg) was dissolved in 1 ml of 2 M NaOH and saponified under argon for 30 min at 35 °C in the dark. The internal standard (*o*-coumaric acid) was added, prior to neutralisation with 2 M HCl and the extraction of the phenolic compounds into ether. The ether phase was evaporated and the residue dissolved in 50/50 water/methanol. 20 µl of this material was injected onto a Purospher C18 column (Merck, Darmstadt, Germany) and a gradient elution was performed using acetonitrile (A) and pH 4.6 sodium acetate buffer (B) at 60 ml/h and 30 °C: (0 min, A = 15 %; 6 min, A = 15 %; 26 mins, A = 35 %; 26.5 min, A = 60 %; 30.5 min, A = 60 %; 31 min, A = 15 %; 35 min, A = 15 %). The eluent was detected at 320 nm. Response factors were determined relative to *o*-coumaric acid (ferulic acid = 0.57 and for the diferulic acids 5-5' = 0.50; 8-O-4' = 1.04; 8-5' cyclic dimer = 1.09 and 8-5' non-cyclic dimer = 0.90).

2.2.5. Physical

High performance size exclusion chromatography (HPSEC) was performed at room temperature on a system consisting of a Shodex OH SB-G guard column (Showa Denko, Tokyo, Japan) followed by in series (Shodex OH-Pak SB-805 HQ and Shodex OH-Pak SB-804 HQ) eluted with 50 mM sodium nitrate buffer containing 0.02 % sodium azide as an antibacterial agent at a flow rate of 42 ml/h. The eluent was detected on-line by:

i. SpectroMonitor 3000 variable wavelength UV detector at 325 nm (LDC/Milton Roy, Paris, France)
ii. MiniDawn light scattering (LS) detector (Wyatt, Santa Barbara, U.S.A.)
iii. T-50A differential pressure viscometer (DPV) (Viscotek, Huston, U.S.A.)

iv. ERC 7515A differential refractometer (RI) (Sopares, Gentilly, France)

Weight average molar masses were calculated using ASTRA (Wyatt, Santa Barbara, U.S.A) and weight intrinsic viscosities using the TRISEC (Viscotek, Huston, U.S.A.). *dn/dc* was taken to be 0.146 ml/g (Chapman, Morris, Selvendran, & O'Neill, 1987; Morris, Foster, & Harding, 2000; Levigne, *et. al*., 2002; Morris, García de la Torre, Ortega, Castille, Smith, & Harding, 2008) for pectin A and its HG and RG-I fractions.

2.2.6. Anion Exchange Chromatography (AEC)

Anion exchange chromatography was performed in order to purify the RG-I fraction on a DEAE-Sepharose CL 6B (Pharmacia) column (30 x 2.6 cm) equilibrated with degassed 0.05 M sodium succinate buffer pH 4.5 at a flow rate of 90 ml/h at room temperature. The hydrolysis products of pectin A after 24 hours incubation with polygalacturonase (APG24) (25 ml) were loaded onto the column and the gel was washed with 400 ml of 0.05 M sodium succinate buffer. The bound material was eluted with a linear NaCl gradient 0→0.4 M NaCl in 0.05 M sodium succinate buffer, 1660 ml). Sodium succinate buffer containing 0.4M NaCl (400 ml) was then applied. Fractions (9 ml) were collected and analysed for their galacturonic acid and neutral sugar contents. Fractions (F1 – F10) were combined and concentrated by in vacuum rotary evaporation at 40 °C.

2.2.7. Preparative Size Exclusion Chromatography (SEC)

Size exclusion chromatography was performed at room temperature on a Sephacryl S-200 column (60 x 1.6 cm) equilibrated with degassed 0.05 M sodium succinate buffer pH 4.5. The anion exchange chromatography fraction containing the RG-I component: APGF10 (1.5 ml) was loaded and eluted at a flow rate of 25 ml/h; 1.5 ml fractions were collected and analysed for their galacturonic acid and neutral sugar contents. Appropriate fractions were combined and concentrated by in vacuum rotary evaporation at 40 °C.

3. Results and Discussion

3.1. Physico-chemical characterisation of pectin A

Pectin A was first characterised/analysed by HPSEC. The peak at ~19 ml (**Figure 2**) is shown to consist entirely of neutral sugars (99 %) – see section **3.3** (**Table 1**). This "component" is "free" from the main pectic chain and constitutes neutral sugar side chains, which were cleaved from the main pectin chain during acid extraction, but are of sufficiently large molar mass to remain after dialysis. Therefore this component has been ignored when calculating the physical and chemical properties of pectin A.

Pectin A exhibits a typical distribution of sugars (**Table 1**), although the amount of total neutral sugars compared to value calculated by Levigne, *et. al*. (2002) appears to be a lot lower due to the removal of the contribution of the "free" neutral sugar side chains.

Acid extracted pectin is heterogeneous with respect to molar mass, intrinsic viscosity and composition (**Tables 1, 2** and **Figure 2**). The use of UV absorbance (325 nm) allows the visualisation of those populations of pectic molecules (or constituents), which are substituted with ferulic acid *i.e.* the arabinan, galactan or arabinogalactan side chains present on the RG-I region of sugar beet pectin. We can see from the differences between the RI and UV profiles that there are distinct populations of pectic molecules; the components eluting at the lowest elution volumes (12 – 14 ml) appear to be richer in ferulic acid than the species, which elute between 14 – 18 ml and should therefore also be richer in neutral sugars. We propose that this neutral sugar/ ferulic acid rich component is the result of dimerisation of pectins via diferulic bridges. Diferulic acids are known to be present in this pectin sample (11 % of total ferulic acids) and have recently been shown to bridge arabinose residues of Driselase® degraded sugar beet pectin (Levigne, *et. al*., 2004a) and to have peripheral locations on pectin hairy regions (Levigne, *et. al*., 2004b).

UV detection also allows a global estimate of the total % of ferulic acid in pectin A, which was found to 0.8 % (**Table 1**), and is in good agreement with the value of 0.7 % found from C18 HPLC after saponification and organic extraction. As ferulic acid only forms ester-linkages with the neutral sugar side chains of the RG-I region, therefore an increase in the % of ferulic acid is

234 therefore the result of a loss of galacturonic acid and can be used as an indication of the purity of
235 the RG-I region.
236
237 **3.2. Enzymatic hydrolysis of pectin A**
238 After 24 hours of enzymatic hydrolysis of pectin A by PG and PME, the weight average molar
239 mass and weight average intrinsic viscosity were reduced considerably as can be seen by the loss
240 of higher molecular weight material this is consistent with previous estimates (Oosterveld,
241 Beldman, & Voragen, 2002) and was confirmed after purification (**Table 1**). As can be seen the
242 peak at 19 ml is unaffected by enzymatic hydrolysis by PG and PME this is consistent with this
243 being a fragment of a neutral sugar side chain and being free of GalA (**Figure 3**). Furthermore
244 100 % of the ferulic acid substituted material is conserved in the pectic fragment at 16-17 ml
245 (excluding the peak at 19 ml) and the amount of ferulic acid has increased to 2.4 %. This is
246 consistent with the purification of the RG-I region of sugar beet pectin, as this is the result of the
247 loss of galacturonic acid and therefore concentration of neutral sugars.
248
249 It is however difficult to calculate the molar masses of the RG-I fragment using light scattering
250 due to the presence of galacturonic acid oligomers (Ralet, *et. al*., 2005b) contributing to the light
251 scattering (LS), refractive index (RI) and differential pressure viscometer (DPV) signals (but not
252 the UV signal), therefore a better estimate of the molar mass and intrinsic viscosity can be made
253 after chromatographic separation on a preparative scale. This will also allow chemical analyses.
254
255 **3.3. Anion exchange chromatography (AEC)**
256 After AEC it can be seen (**Figure 4**) that there are 10 components most of which (F2 – F9) are
257 oligogalacturonides of various DPs (1 – 10), DMs and DAcs (see Ralet, *et. al*., 2005a for a full
258 characterisation). The unbound fraction (F1) is the neutral sugar fraction (99 %) described
259 earlier. The fraction, which is retained the longest (F10) is richer in neutral sugars and is
260 presumed to be the RG-I fraction, although it has been shown previously (Ralet, *et. al*., 2005a)
261 that this fraction also contains larger oligogalacturonides.
262
263

3.4. Preparative Size Exclusion Chromatography (SEC)

This fraction (F10) was subjected to SEC on Sephacryl S-200 (**Figure 5**) and two sub-fractions (F10a and F10b) were separated. The higher molecular weight the sub-fraction (F10a) originates from the RG-I region and the lower molecular weight sub-fraction consists of oligogalacturonides (Ralet, *et. al*., 2005a). Fraction F10a which has not previously been examined in detail, was dialysed, freeze-dried and physico-chemical properties characterised.

3.5. Physico-chemical characterisation of RG-I fraction (APG24F10a)

The RG-I region was found to contain 22 mol% GalA; 17 mol% Rha and 62 mol% neutral sugars (GalA: Rha: NS ratio of 1.3: 1.0: 3.6) suggesting that this fraction is indeed the RG-I region and the HG component has been almost entirely degraded, this was also supported by a further increase in the amount of ferulic acid to 2.4 %, which is 3 times that present in pectin A and is slightly larger than the increase in total neutral sugars ~ 2.6 times (**Table 1**). This fraction was found to have a weight average molar mass of 188000 g/mol and the weight average intrinsic viscosity is 36 ml/g (**Table 2**), which is relatively low for a molecule of this molar mass and is reflective of a highly compact or random coil conformation. This value is much higher than previous estimates of 50000 g/mol (Oosterveld, *et. al*., 2002) and 12000 g/mol (Guillon, & Thibault, 1990) calculated from light scattering and viscosity measurements respectively, but it should be noted that in both cases the values are likely to be underestimated due to the presence of contaminants *e.g.* galacturonic acid oligomers, salts and enzymes. In our case the weight average molar mass of RG-I is greatly influenced by presence of "aggregates" appearing as shoulders on the UV chromatogram at 13 – 14 and 14 – 15 ml (**Figure 3**), the exact nature of these aggregates is not clear, but we believe that any diferulic bridges present in the pectin A would also be present in the purified RG-I region as enzymatic hydrolysis and chromatographic separation appear to have no affect on ferulic acid residues. Another factor influencing the molar mass is that at least some of the lower molar mass RG-I molecules may have co-eluted with the larger oligogalacturonides during SEC fractionation.

293 **3.6. Physical characterisation of HG fraction (AHCl72)**

294 During 72 hours acid hydrolysis of pectin A at 80 °C a precipitate is formed (~ 98 % GalA) this
295 is consistent with previous measurements (Thibault, *et. al*., 1993). After neutralisation both the
296 insoluble and soluble fractions were characterised by HPSEC. The soluble fraction did not
297 consist of any polymeric material; all components eluted near the total volume (results not
298 shown) and probably contained fragments of neutral sugar side chains. The insoluble fraction
299 also contains low molar mass material eluting near the total volume and represent the salts
300 formed after neutralisation. There is however a polymeric component with a peak at an elution
301 volume at ~17 ml (x in **Figure 3**). This component was found to have a weight average molar
302 mass of 20000 g/mol (**Table 2**). This value is good agreement with the values of 17000 – 21000
303 g/mol estimated previously for an acid hydrolysed beet pectin HG region (Thibault, *et. al*., 1993;
304 Hellin, Ralet, Bonnin, & Thibault, 2005; Yapo, Lerouge, Thibault, & Ralet, 2007; Ralet, *et al*.,
305 2008b) and slightly higher than the estimated molar mass of 16000 g/mol for an enzymatically
306 hydrolysed sugar beet pectin HG region (Bonnin, Dolo, Le Goff, & Thibault, 2002b). The
307 weight average intrinsic viscosity of 77 ml/g (**Table 2**) is again in general agreement with value
308 found previously (75 - 92 ml/g) for the homogalacturonan region of pectins (Thibault, *et. al*.,
309 1993; Yapo, *et. al.* 2007; Ralet, *et al*., 2008b).
310
311 **3.7. Conformational analysis**
312 **3.7.1. Global analysis method (HYDFIT)**
313 The linear flexibility of polymer chains can also be represented quantitatively in terms of the
314 persistence length, L_p of equivalent *worm-like chains* (Kratky, & Porod, 1949) where the
315 persistence length is defined as the average projection length along the initial direction of the
316 polymer chain. In the case of a theoretical perfect random coil $L_p = 0$ and for the equivalent
317 extra-rigid rod (Harding, 1997) $L_p = \infty$, although in practice limits of ~ 1 nm for random coils
318 (*e.g.* pullulan) and 200 nm for a extra-rigid rod (*e.g.* DNA) are more appropriate (Tombs, &
319 Harding, 1998).
320
321 The persistence length, L_p and mass per unit length, M_L can be estimated using Multi-HYDFIT
322 program (Ortega, & García de la Torre, 2007) which considers data sets of intrinsic viscosity and
323 molar mass. It then performs a minimisation procedure (see Ortega, & García de la Torre, 2007)

finding the best values of M_L and L_p satisfying the Bushin-Bohdanecky (Bushin, Tsvetkov, Lysenko, & Emel'yanov, 1981; Bohdanecky, 1983) equation (equation 1).

$$\left(\frac{M_w^2}{[\eta]}\right)^{1/3} = A_0 M_L \Phi^{-1/3} + B_0 \Phi^{-1/3} \left(\frac{2L_p}{M_L}\right)^{-1/2} M_w^{1/2} \quad (1)$$

and

$$M_L = \frac{m}{l} \quad (2)$$

m and l are the average molar mass and length of the average monomeric unit.

From **Figures 6A-C** and **Table 2** we can see that the persistence lengths for pectin A and the HG fraction are larger than that of the RG-I fraction. This would suggest a random coil conformation for the RG-I region and a less flexible conformation for both pectin A and HG regions. These results are consistent with previous findings which suggest that sugar beet; apple and flax pectins have flexible structures (Ralet, *et al.*, 2008b; Axelos, & Thibault, 1991; Cros, *et al.*, 1996) and that HG–rich regions (or pectins) are considerably stiffer (Cros, Garnier, Axelos, Imbery, & Perez, 1996; Braccini, Grasso, & Perez, 1999; Noto, Martorana, Bulone, & San Biagio, 200; Morris, *et al.*, 2008). The values of the mass per unit lengths (**Figures 6A-C** and **Table 2**) indicate that both sugar-beet pectin A and the RG-I fraction are highly branched (hairy) and the HG region is essentially unbranched (smooth), although we would expect the value for the RG-I region to be higher than that of pectin A. Overall flexibility can be estimated from the ratio of L_p/M_L ($nm^2 mol/ g$) which decreases with increasing flexibility (Patel, Morris, Ebringerová, Vodenicarová, Velebny, Ortega, García de la Torre, & Harding, 2008). Therefore we can see that in terms of flexibility RG-I > pectin A > HG ≈ citrus pectin (**Table 2**).

3.7.2. Conformation zoning (Normalised scaling relations)

Pavlov, *et. al.* (1999) described a new procedure to represent the conformation of polymers in solution based on the relationship between their molar mass, intrinsic viscosity and mass per unit length, M_L. In this case we have taken the mass per unit length calculated previously using the HYDFIT algorithm (Ortega, & García de la Torre, 2007). As we can see from **Figure 7** both

353 pectin A and the HG fraction have conformations which fall in either the rigid rod or semi-
354 flexible coil zones this is in agreement with findings on citrus pectins (Morris, *et. al.*, 2008) (N. B.
355 the high value of M_L for pectin A may lead to an overestimation of the rigidity). Whereas the
356 RG-I region clearly adopts a random coil conformation, which is consistent with a persistence
357 length of 1.4 nm (Tombs, & Harding, 1998). This is again consistent with a more flexible
358 structure for the RG-I fraction.
359

4.Conclusions

Acid extracted sugar beet pectin was shown to be heterogeneous in terms of composition and it is proposed that a high molar mass ferulic acid-rich fraction may be the result of diferulic acid bridging between pectin molecules and more specifically between the RG-I regions of pectin molecules.

The enzymatic treatment of pectin A followed by AEC and SEC resulted in a RG-I fraction which was now free of the HG component. This RG-I fraction was shown to be of high weight average molar mass (188000 g/mol), but low intrinsic viscosity (36 ml/g), which is consistent with a random coil conformation. This RG-1 fraction has a GalA: Rha ratio of 1.3: 1.0 and is composed of almost 80 % neutral sugars and 2.4 % ferulic acid.

The HG fraction of acid extracted pectin was also characterised, although without further purification and was found, to have a relatively low weight average molar mass (20000 g/mol), but a rather high intrinsic viscosity (77 ml/g), which is consistent with previous molar mass and intrinsic viscosity estimates (Thibault, *et. al.*, 1993; Bonnin, *et. al.*, 2002b; Ralet, *et al.*, 2008b) and with the HG region being rigid in solution.

In general we can conclude that the degradation of the HG region(s) has an important impact on intrinsic viscosity, but less on molar mass and the inverse is true for the degradation of RG-I region.

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521

522 **Table 1** Chemical composition of pectin A and its RG-I fraction

Sample	Sugar composition, mol %			Ferulic acid content, %[1]
	Gal A	Rha	Other neutral sugars	
Pectin A[2]	68.6	8.4	22.2	0.8
APG24F1 (Pectin A neutral sugar fraction)	n.d.	n.d.	99	2.0
APG24F10a (RG-I fraction)	21.9	16.7	61.5	2.4

523

524 [1]% FA = 100 % x ($UV_{calculated\ mass}$/ $RI_{calculated\ mass}$)

525 [2]does not include the peak shown to consist almost entirely (99 %) of neutral sugars *i.e.*
526 APG24F1 after anion exchange chromatography

527

528 N.B. For the chemical composition of low molecular weight enzymatic hydrolysis products
529 (APG24F2 - APG24F9) see Ralet, *et. al*. (2005a)

530
531

532 **Table 2** Physical and conformational properties of sugar beet pectin A and its HG and RG-I
533 fractions. Values for a "typical" low methoxyl citrus pectin (P_{19}) are shown for comparison[1].

Sample	M_w, g/ mol	$[\eta]_w$, ml/ g	M_L, g/ mol nm	L_p, nm	L_p/M_L, nm^2mol/ g
A[2]	286000	285	620	7.3	0.0118
RG-I	188000	36	555	1.4	0.0025
HG	20000	77	395	9.8	0.0248
P_{19}	165000	395	330	10.0	0.0303

534

535 [1]from Morris, *et al*., 2008

536 [2]does not included the peak shown to consist entirely of neutral sugars *i.e*. APG24F1 after anion
537 exchange chromatography

538
539

Legends to figures

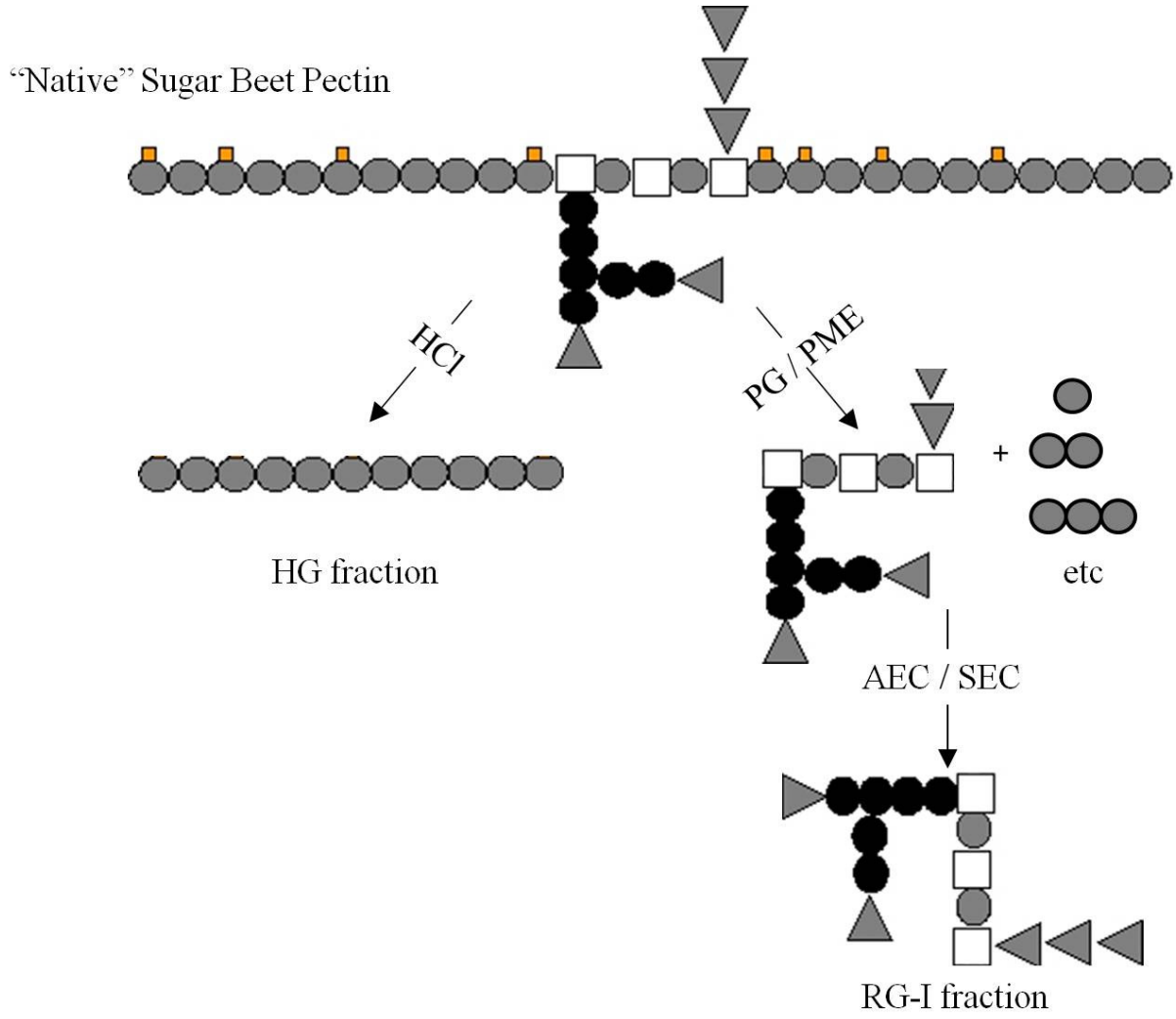
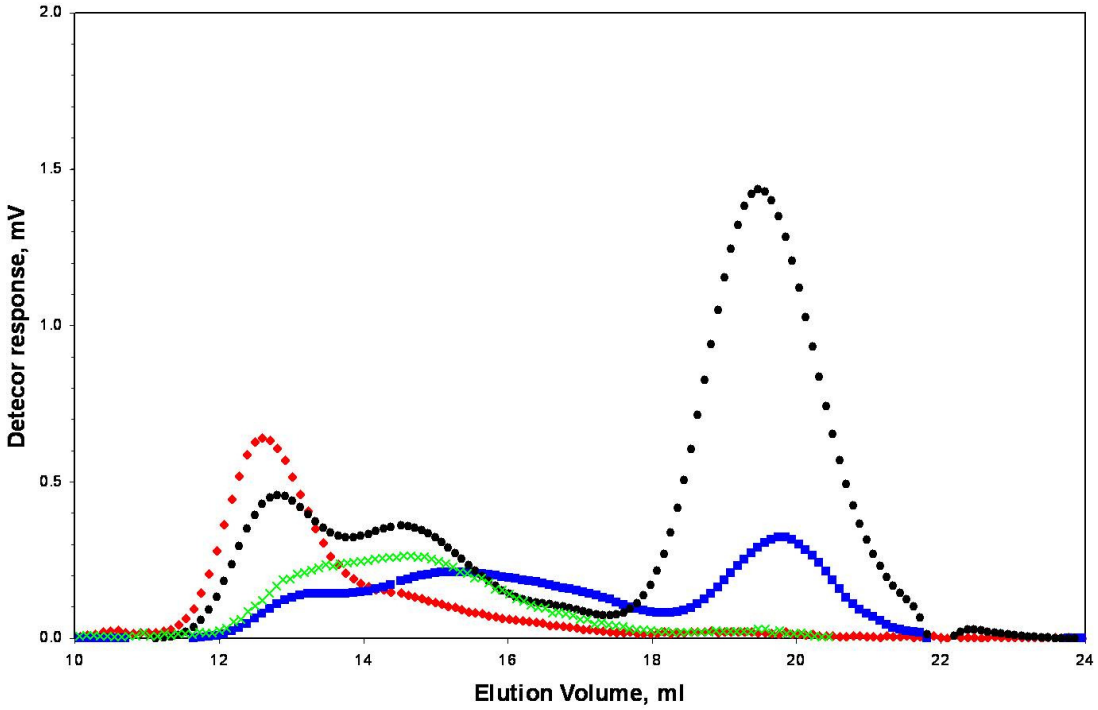


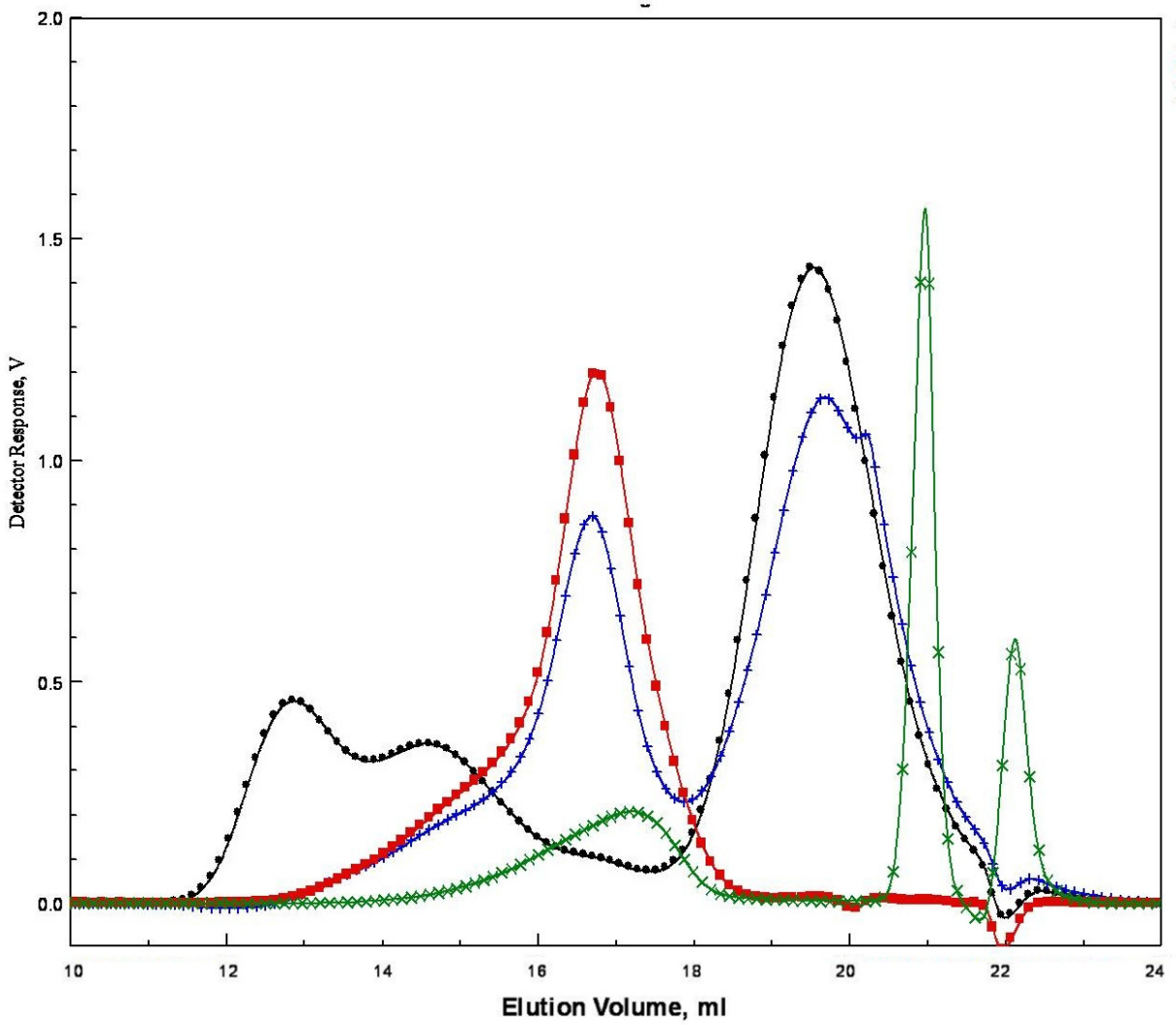
Figure 1 - Schematic structure for generalised sugar beet pectin: galacturonic acid (●); galactose (●); arabinose (▼); rhamnose (□) and methyl groups (▪). Adapted from Perez et al., 2003. PG - endopolygalacturonase I and II from *A. niger*; PME - fungal pectin methyl esterase from *A. aculeatus*; AEC – anion exchange chromatography and SEC – size exclusion chromatography.



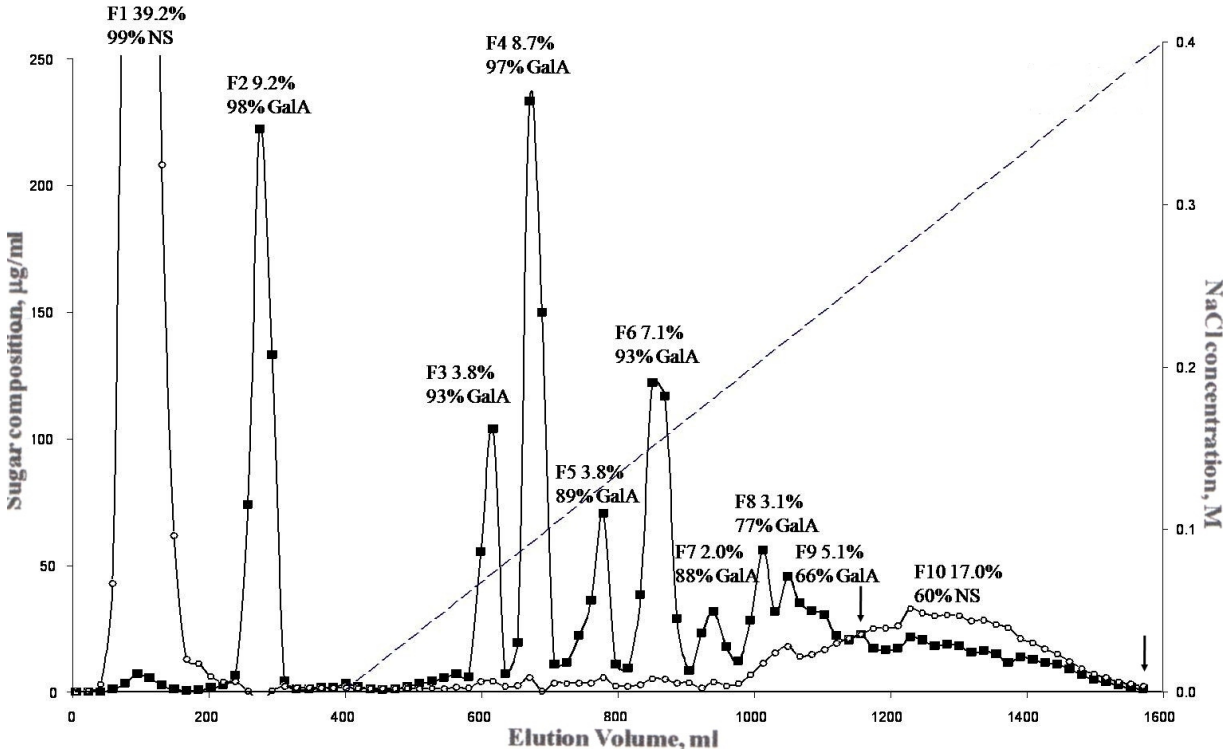
547

548 **Figure 2** - Multi-detector HPSEC chromatogram for pectin A: LS 90° (♦), RI (■), UV 325 nm
549 (•) and DPV (x). N. B. The LS 90 ° and DPV signals have been multiplied by 5 to improve
550 visualisation.

551



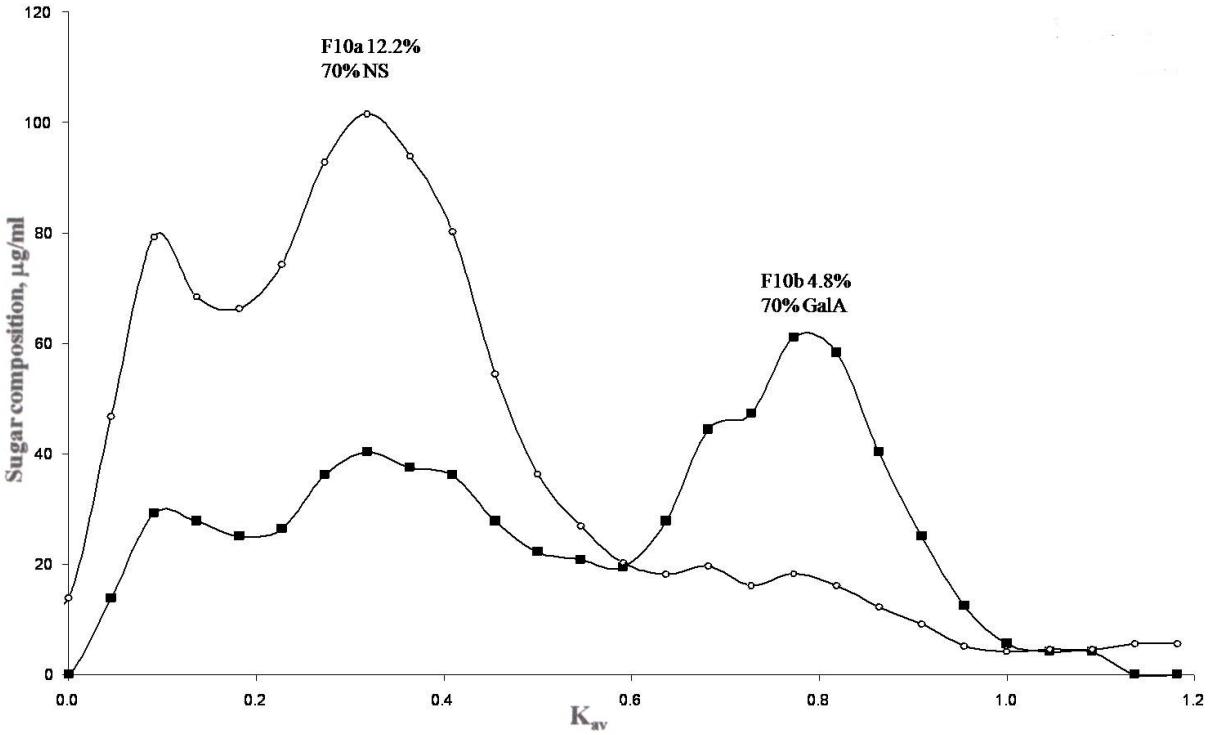
552
553 **Figure 3** - HPSEC chromatogram for Pectin A (•), APG24 (+), APG24F10a (■) and AHCl72
554 (x). N.B. The chromatogram for AHCl72 is an RI trace; all others are UV absorbance at 325nm.
555 AHCl72 has contains no ferulic acid and therefore shows no UV signal at 325nm.
556



557

558 **Figure 4 -** Anion exchange chromatogram (DEAE-Sepharose CL 6B) for pectin A after
559 enzymatic hydrolysis for 24 hours at 30 °C with f-PME, PGs I and II (-■- Gal A and -○- neutral
560 sugars). The proportion of each fraction present is indicated, together with the major constituent:
561 galacturonic acid (GalA) or total neutral sugars (NS). Fraction APGF10 which will be further
562 purified by SEC is indicated by the arrows.

563



564

565 **Figure 5 -** Size exclusion chromatogram (Sephacryl S-200) for APGF10 (-■- Gal A and -○-

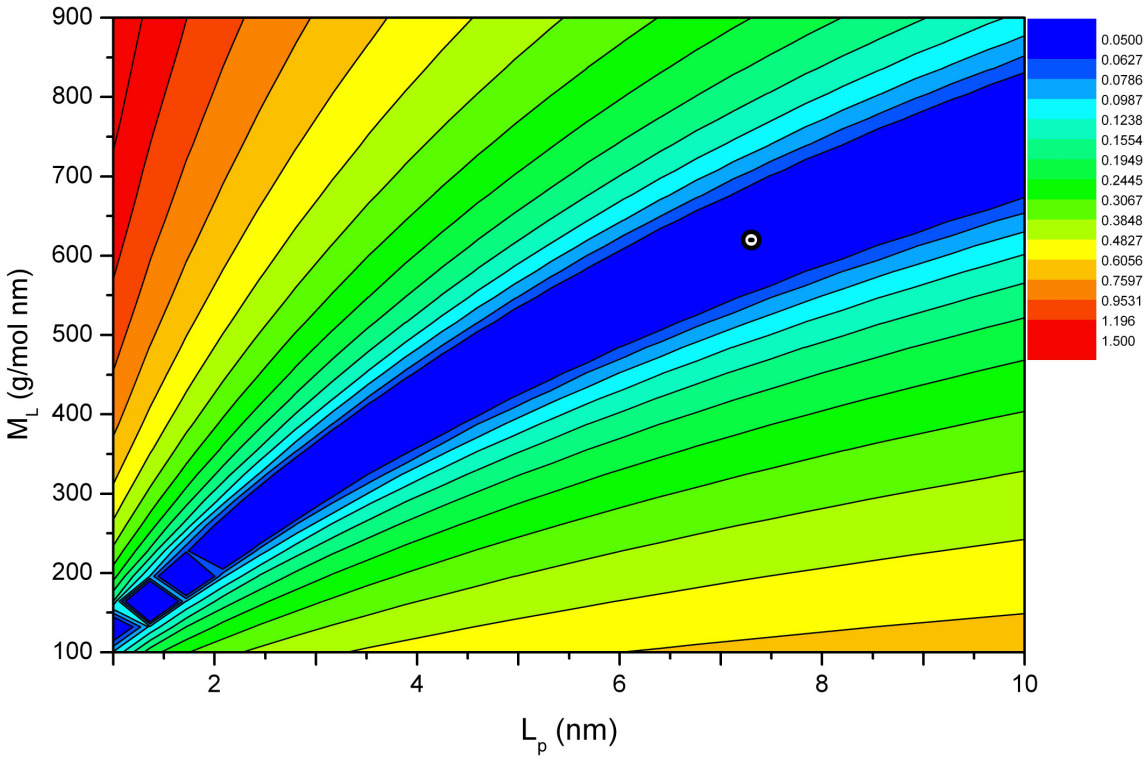
566 neutral sugars). The proportion of each fraction present is indicated, together with the major

567 constituent: galacturonic acid (GalA) or total neutral sugars (NS).

568

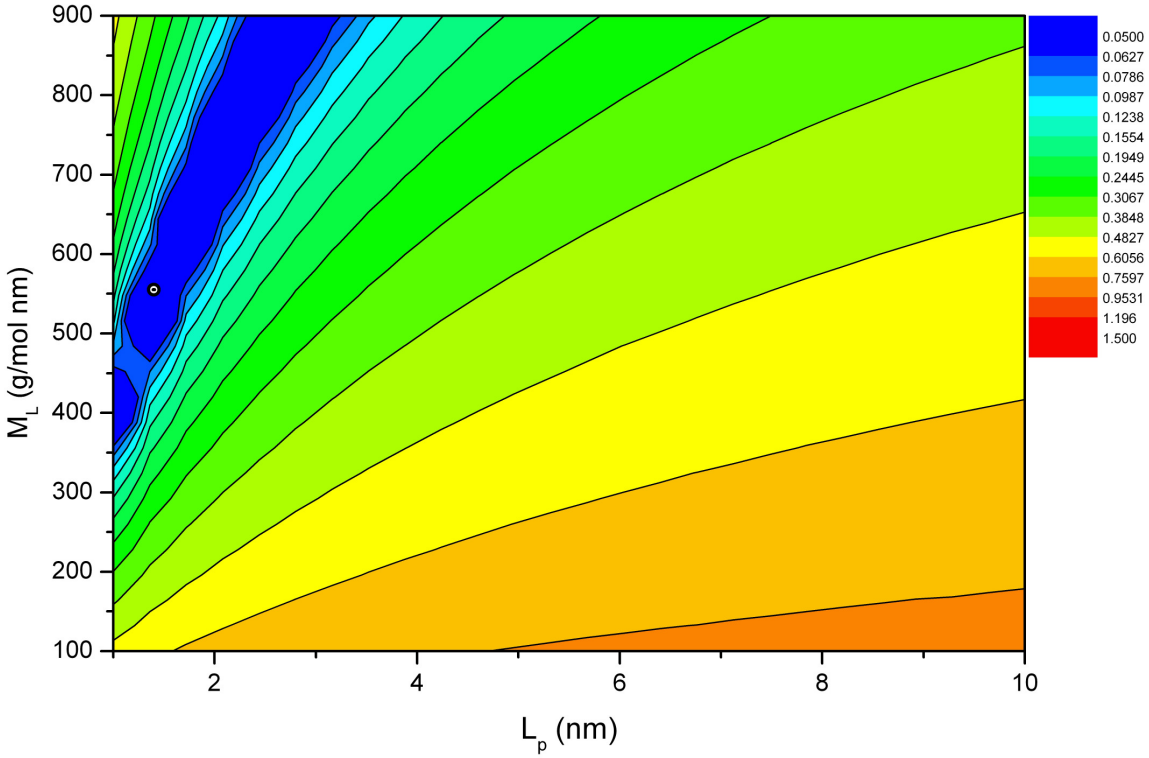
569

570 **Figure 6** - Solutions to the Bushin-Bohdanecky using the HYDFIT algorithm (Ortega and García
571 de la Torre, 2007). The x-axis and y-axis represent L_p (nm) and M_L (g/ mol nm) respectively.
572 The target function, Δ is calculated over a range of values for M_L and L_p. In these
573 representations, the values of Δ function are represented by the full colour spectrum, from the
574 minimum in the target function in blue ($\Delta = 0.5$) to red ($\Delta \geq 1.5$). The calculated minima are
575 indicated (⊙).
576



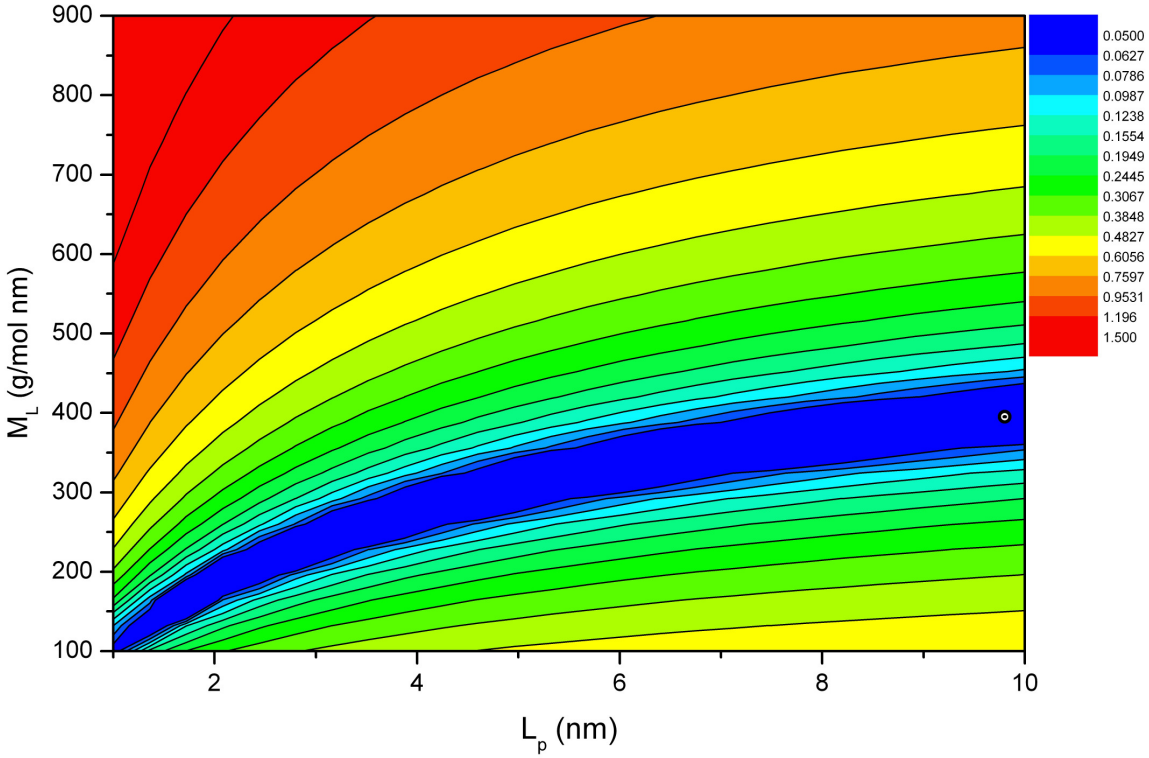
577
578 **A** - Pectin A ($L_p = 7.3$ nm and $M_L = 620$ g/ mol nm)

579



580
581 **B** - RG-I fraction (L_p = 1.4 nm and M_L = 555 g/ mol nm)

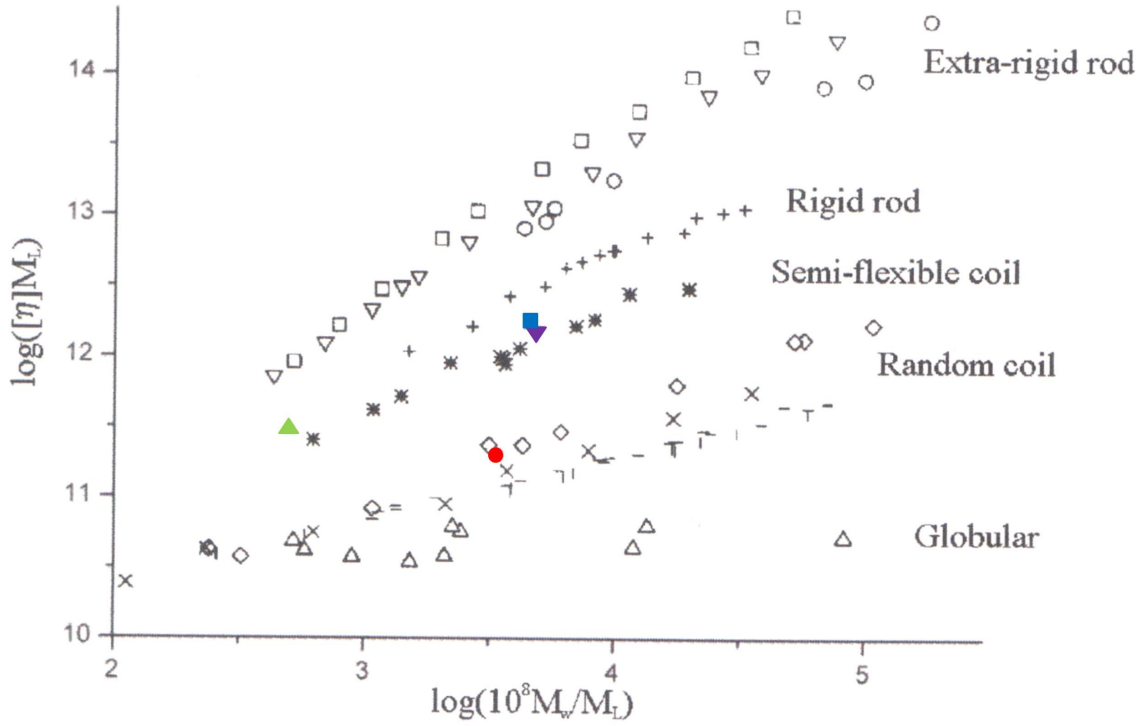
582



583

584 **C** - HG fraction (L_p = 9.8 nm and M_L = 395 g/ mol nm)

585



586
587 **Figure 7 -** Normalised scaling plot of $[\eta]M_L$ versus M_w/M_L (adapted from Pavlov, *et. al.*, 1999)
588 where the solution conformations for Pectin A (■) and its RG-I (●) and HG (▲) fractions are
589 indicated a typical citrus pectin is shown for comparison (▼). All other symbols are as defined
590 previously (Pavlov, *et. al.*, 1999) in brief: schizophyllan (□), DNA (○), globular proteins (Δ),
591 xanthan (∇), poly(1-vinyl-2-pyrrolidone) (◊), cellulose nitrate (+), pullulan (x), methyl cellulose
592 (*), poly-α-methylstyrene (-) and polystyrene (|).